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LncRNA DSCAM-AS1 promoted cell proliferation and invasion in osteosarcoma by sponging miR-101

C.-L. YU, N.-W. XU, W. JIANG, H. ZHANG, Y. MA

Department of Spinal Surgery, the Affiliated Changzhou No. 2 People's Hospital of Nanjing Medical University, Changzhou, P.R. China

Changlin Yu and Nanwei Xu contribute equally to this research and should be considered as co-first authors

Abstract. – OBJECTIVE: Long noncoding RNAs (lncRNAs) play critical roles in osteosarcoma (OS) progression. LncRNA DSCAM-AS1 has been reported to function as a tumor promoter in various cancers. However, the potential mechanism of DSCAM-AS1 in OS remains rarely know.

PATIENTS AND METHODS: The expression levels of DSCAM-AS1 and miR-101 were detected by RT-qPCR. The correlation between DSCAM-AS1 and miR-101 expression was analyzed by Pearson's correlation. Kaplan-Meier analysis was used to assess the overall survival rate. Cell viability and invasion were assessed by MTT assay and transwell assays, respectively. A Luciferase reporter assay was used to identify the relationship between DSCAM-AS1 and miR-101.

RESULTS: In the present study, it was demonstrated that DSCAM-AS1 expression was significantly upregulated in OS tissues and cells and high expression of DSCAM-AS1 predicted poor prognosis in OS patients. In addition, the silencing of DSCAM-AS1 suppressed the viability and invasion of OS cells, while DSCAM-AS1 overexpression promoted cell viability and invasion. Furthermore, we found that DSCAM-AS1 inhibited miR-101 expression by direct interaction and DSCAM-AS1 promoted OS progression by sponging miR-101. In addition, miR-101 expression was negatively correlated with DSCAM-AS1 expression. Patients with low miR-101 expression had a shorter overall survival time compared with those with high miR-101 expression.

CONCLUSIONS: The present study demonstrated that DSCAM-AS1 accelerated OS cell progression by sponging miR-101, which might provide a new sight in the treatment of OS.

Key Words:
DSCAM-AS1, MiR-101, Osteosarcoma.

Introduction

Osteosarcoma (OS) is one of the most common bone malignancy in children and adolescents[1]. Although great improvements have been made in diagnosis and surgical treatment, the overall survival rate remained unsatisfactory due to recurrence or metastasis[2,3]. In the past years, lncRNA was found to act as a crucial role in tumor progression[4], which provided a new study direction for OS treatment.

Long noncoding RNAs (lncRNAs) are a class of non-coding transcripts with a length longer than 200 nts[5,6]; they were involved in the progression of human malignancies, such as colorectal cancer[7], prostate cancer[8], and osteosarcoma[9]. A newly discovered lncRNA DSCAM-AS1 was found to act as an oncogene in several cancers. Of note, lncRNA DSCAM-AS1 promoted the tumorigenesis of colorectal cancer by regulating miR-144-5p/CDKL1[10]. LncRNA DSCAM-AS1 targeted BCL11A to accelerate non-small cell lung cancer progression[11]. However, the potential regulatory mechanism of DSCAM-AS1 in OS was rarely understood.

MicroRNAs (MiRNAs) are a type of small noncoding RNAs with a length of 21 to 23 nts, which regulated gene expression at the post-transcriptional level[12,13]. An increasing body of evidence has revealed that miRNAs participated in the tumorigenesis of various cancers, including OS. MiR-20a accelerated OS cell proliferation by upregulating TAK1[14]. MiR-411 inhibited MTSS1 expression to facilitate OS progression[15]. Several researches revealed that miR-101 functioned as a tumor

Corresponding Author: Yong Ma, MD; e-mail; yongma0101@163.com

suppressor in OS progression. Jiang et al[16] discovered that miR-101 attenuated OS cell proliferation and invasion by modulating ROCK1. Zhang et al[17] demonstrated that miR-101 suppressed the OS cells metastasis of by decreasing EZH2 expression. However, the regulatory mechanisms of miR-101 in OS remains to be further clarified.

In our study, we demonstrated that DSCAM-AS1 promoted OS cell viability and invasion by targeting miR-101. This finding might provide a novel theoretical basis for the targeted therapy of OS.

Patients and Methods

Clinical Specimens

OS tissues and adjacent normal tissues were obtained from 32 OS patients in Changzhou Second People's Hospital Affiliated to Nanjing Medical University. The inclusion criteria for the study were as follows: i) patients diagnosed with osteosarcoma for the first time; ii) the patient did not receive treatment before admission. Exclusion criteria: i) previous history of malignant tumor; ii) patients who received treatment within 3 months before admission. Written informed consent was obtained from all patients. This work was approved by the Ethics Committee of Changzhou Second People's Hospital Affiliated to Nanjing Medical University. Tissues obtained after resection were stored in liquid nitrogen at -80°C for further use.

Cell Lines and Cell Culture

Human osteoblast cells (hFOB) and OS cell lines (U2OS, SAOS2 and HOS) were attained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) with fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C and 5% CO_2.

Cell Transfection

The short hairpin RNAs against DSCAM-AS1 (shDSCAM-AS1) with its negative control (shNC), miR-101 mimics with it negative control (NC mimics), miR-101 inhibitor with its negative control (NC inhibitor), and pcDNA3.1/DSCAM-AS1 with empty pcDNA3.1 as control were purchased from GenePharma (Shanghai, China). The transfection was conducted with Lipofectamine 2000 reagent (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) Assay

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, cDNA was synthesized by using PrimeScript RT reagent kits (TaKaRa, Dalian, China). PCR amplification was applied using the SYBR Green Real-Time PCR Kit (TaKaRa, Dalian, China). GAPDH and U6 served as internal controls. The results were analyzed using the $2^{-\Delta\Delta CT}$ method. The primers were presented as follows: DSCAM-AS1 forward, 5′-GCCCTGCCTTCAGCCTCCTCA-3′ and reverse, 5′- GAAACGTCGGCCTGGCCTTGT-3′; miR-101 forward, 5′-TGCGGCAGTGGTTTTACCCTATG-3′ and reverse, 5′-CCAGTGCAGGGTCCGAGGT-3′; GAPDH forward, 5'-ATGGAGAAGGCTGGGGCTC-3' and reverse, 5'-AAGTTGTCATGGATGACCTTG-3'; and U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'.

Dimethyl Thiazolyl Diphenyl Tetrazolium (MTT) Assay

The MTT assay was utilized to assess cell viability. The cells were seeded in a 96-well plate cultured overnight. Next, 10 μL MTT (Bioswamp, Wuhan, China) was added and then cells were cultured at 37°C for 4 hours. The absorbance was examined at 450 nm utilizing a microplate reader (Bio-Rad, Hercules, CA, USA).

Transwell Assay

Cell invasion was detected by using transwell chambers (8.0 μm pore size; BD Biosciences, Franklin Lakes, NJ, USA). Transfected cells in serum-free RPMI-1640 medium were seeded into the upper chamber pre-coated with Matrigel (BD, Franklin Lakes, NJ, USA). 550 μl RPMI-1640 medium (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% FBS was added in the lower chamber. After incubated for 48 hours, and the cells in the lower chamber were fixed and dyed with crystal violet. Finally, invaded cells were counted under a microscope (Olympus Corp, Tokyo, Japan).

Luciferase Reporter Assay

The wild-type and mutant-type of the DSCAM-AS1 sequences were subcloned into the pmirGLO

reporter vectors (Promega, Madison, WI, USA) to construct DSCAM-AS1-Wt and DSCAM-AS1-Mut vectors. Then, these constructed vectors were co-transfected with NC mimics, miR-101 mimics, NC inhibitor or miR-101 inhibitor into OS cells. After 48 h, the luciferase activity was assessed using the Dual-Luciferase reporter system (Promega, Madison, WI, USA).

Statistical Analysis

Data were expressed as the mean ± standard deviation (SD). SPSS 19.0 (SPSS Corp., Armonk, NY, USA) and GraphPad Prism 6.0 (La Jolla, CA, USA) were employed for statistical analysis. Comparisons between two groups were performed by a Student's *t*-test. Comparisons among three groups were analyzed using one-way ANOVA followed by Tukey's test. Pearson's correlation analysis was employed to analyze the relationship between DSCAM-AS1 and miR-101 expression. The overall survival of OS patients was analyzed by Kaplan-Meier method and log-rank test. $p<0.05$ indicated statistically significant.

Results

DSCAM-AS1 Was Upregulated in OS and Predicted Poor Prognosis

We first detected the DSCAM-AS1 expression in OS tissues, and the results indicated that DSCAM-AS1 expression was dramatically increased in OS tissues (Figure 1A). Next, RT-qPCR results revealed that the expression of DSCAM-AS1 was significantly higher in OS cell lines (U2OS, SAOS2, and HOS) than that in human osteoblast cells (hFOB) (Figure 1B). Moreover, Kaplan-Meier analysis showed that patients with high DSCAM-AS1 expression exhibited a worse prognosis compared with patients with low DSCAM-AS1 expression (Figure 1C). Furthermore, it was found that DSCAM-AS1 expression was positively correlated with TNM stage, lymph node metastases, and distant metastases, while no correlation was found between the DSCAM-AS1 expression and age or sex (Table I). Taken together, these data indicated that DSCAM-AS1 was upregulated in OS, and high expression of DSCAM-AS1 predicted poor prognosis in patients.

DSCAM-AS1 Accelerated OS Progression

To explore the functional role of DSCAM-AS1 in OS cells, shDSCAM-AS1 and shNC were transfected into U2OS cells, and pcDNA3.1 and pcDNA3.1/DSCAM-AS1 were transfected into SAOS2 cells. The results demonstrated that DSCAM-AS1 expression was decreased after transfected with shDSCAM-AS1 in U2OS cells, while increased in SAOS2 cells transfected with pcDNA3.1/DSCAM-AS1(Figure 2A). MTT and transwell assays revealed that cell viability and

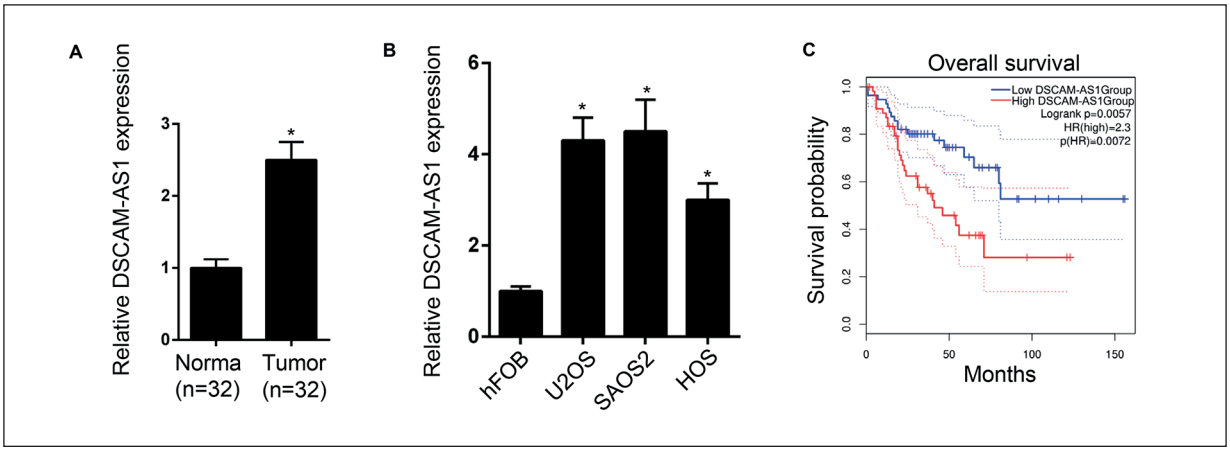

Figure 1. DSCAM-AS1 was upregulated in OS and predicted poor prognosis. **A,** RT-qPCR showed the relative DSCAM-AS1 expression in OS tissues (n=32) and normal tissues (n=32). **B,** The expression of DSCAM-AS1 in OS cell lines (U2OS, SAOS2, MG63, HOS) and human osteoblast cells (hFOB) was measured by RT-qPCR. **C,** Kaplan-Meier survival analysis showed the correlation between DSCAM-AS1 expression and prognosis of OS patients. *$p<0.05$.

Table I. Correlation between DSCAM-AS1 expression and clinicopathologic features in patients with OS.

Clinical Features	Total	DSCAM-AS1		*p*-value
		High	Low	
Age (years)				0.587
< 20	15	8	7	
≥ 20	17	10	7	
Gender				0.475
Male	18	8	10	
Female	14	8	6	
TNM stage				0.006
I-II	13	5	8	
III-IV	19	14	5	
Distant metastasis				0.007
Absent	19	5	14	
Present	13	9	4	
Lymph node metastasis				0.005
Positive	22	15	7	
Negative	10	5	5	

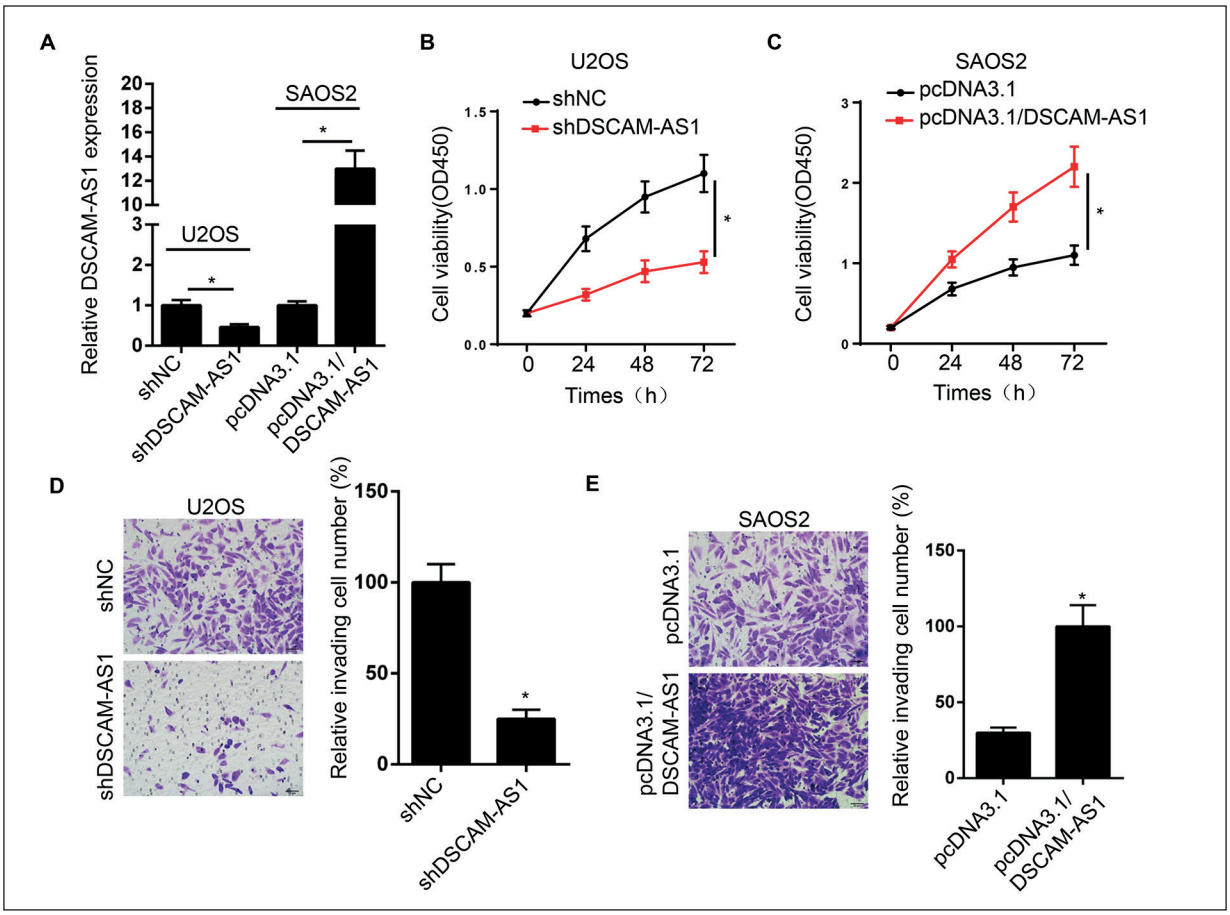

Figure 2. DSCAM-AS1 accelerated OS progression. **A,** RT-qPCR showed the transfection efficiency of shDSCAM-AS1 or pcDNA3.1/DSCAM-AS1 in U2OS or SAOS2 cells. **B,** MTT assay showed the cell viability of U2OS transfected with shNC and shDSCAM-AS1. **C,** MTT assay showed the cell viability of SAOS2 transfected with pcDNA3.1 and pcDNA3.1/DSCAM-AS1. **D,** Transwell assay showed the invasion ability of U2OS transfected with shNC and shDSCAM-AS1 (magnification ×40). **E,** Transwell assay showed the invasion ability of SAOS2 transfected with pcDNA3.1 and pcDNA3.1/DSCAM-AS1 (magnification ×40). **p*<0.05.

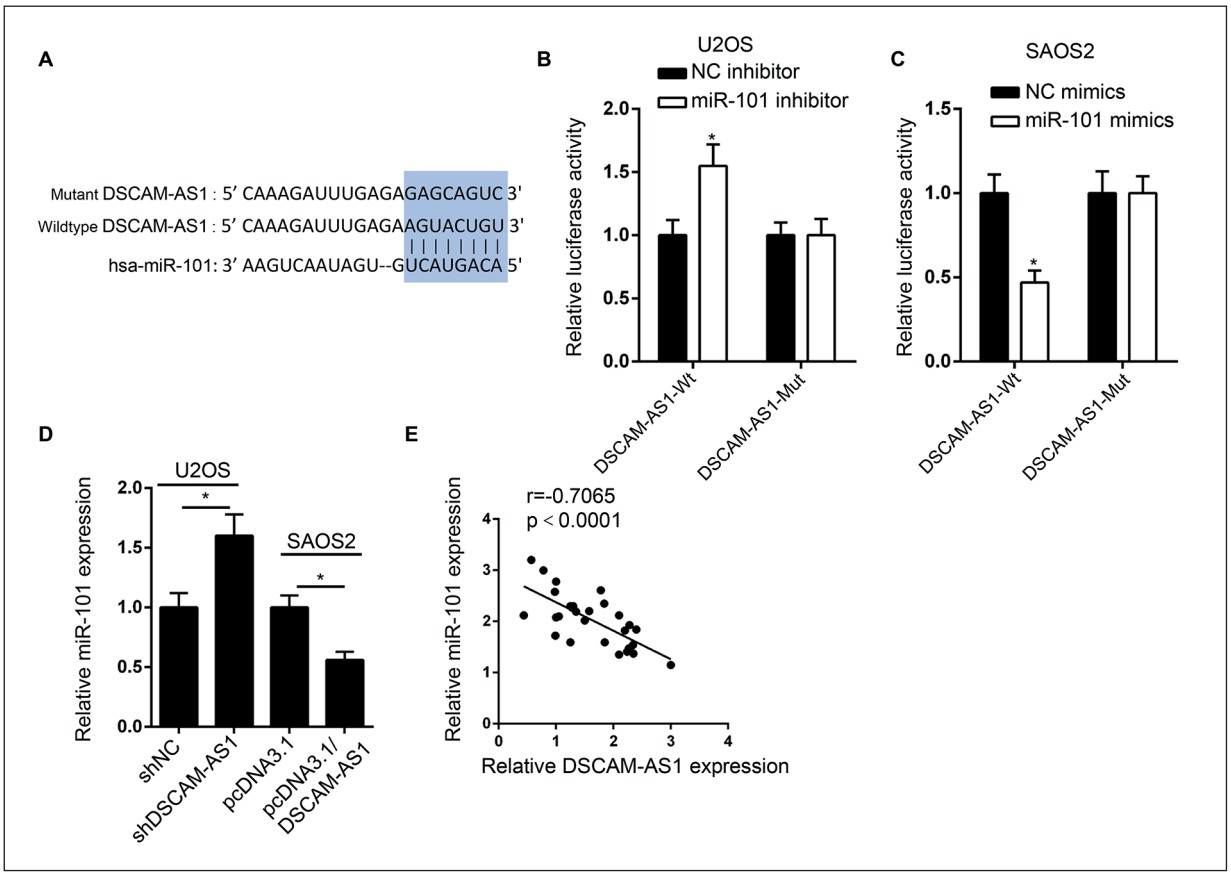

Figure 3. MiR-101 was a target of DSCAM-AS1 in OS. **A,** StarBase dataset showed the binding sites between DSCAM-AS1 and miR-101. **B,** Luciferase reporter assay showed luciferase activity in U2OS cells transfected with NC inhibitor and miR-101 inhibitor. **C,** Luciferase reporter assay showed luciferase activity in SAOS2 cells transfected with NC inhibitor and miR-101 mimics. **D,** RT-qPCR showed the relative miR-101 expression in OS cells transfected with shNC and shDSCAM-AS1 or pcDNA3.1, pcDNA3.1/DSCAM-AS1. **E,** Pearson analysis showed the correlation of DSCAM-AS1 expression with miR-101 expression in OS tissues. *p<0.05.

invasion were markedly attenuated by silencing of DSCAM-AS1 in U2OS cells but promoted by DSCAM-AS1 overexpression in SAOS2 cells (Figure 2B-E). Therefore, we concluded that the knockdown of DSCAM-AS1 inhibited cell viability and invasion in OS.

MiR-101 Was a Target of DSCAM-AS1 in OS

As shown in Figure 3A, starBase website was used to predict the binding site of miR-101 on DSCAM-AS1. Luciferase assay demonstrated that miR-101 inhibitor increased the Luciferase activity of DSCAM-AS1-Wt in U2OS cells and miR-101 mimics decreased DSCAM-AS1-Wt Luciferase activity in SAOS2, while miR-101 inhibitor or mimic had no impacts on the luciferase activity of DSCAM-AS1-Mut (Figure 3B and C). Following that, RT-qPCR results showed that knockdown of DSCAM-AS1 increased the expression of miR-101 in U2OS cells, while its expression was decreased in SAOS2 cells by DSCAM-AS1 overexpression (Figure 3D). In addition, Pearson analysis indicated that DSCAM-AS1 expression had a negative correlation with miR-101 expression in OS tissues (Figure 3E). These findings showed that DSCAM-AS1 directly bound with miR-101.

DSCAM-AS1 Promoted OS Progression by Sponging MiR-101

To further investigated whether DSCAM-AS1 promoted OS progression by regulating miR-101, a series of rescue experiments were conducted.

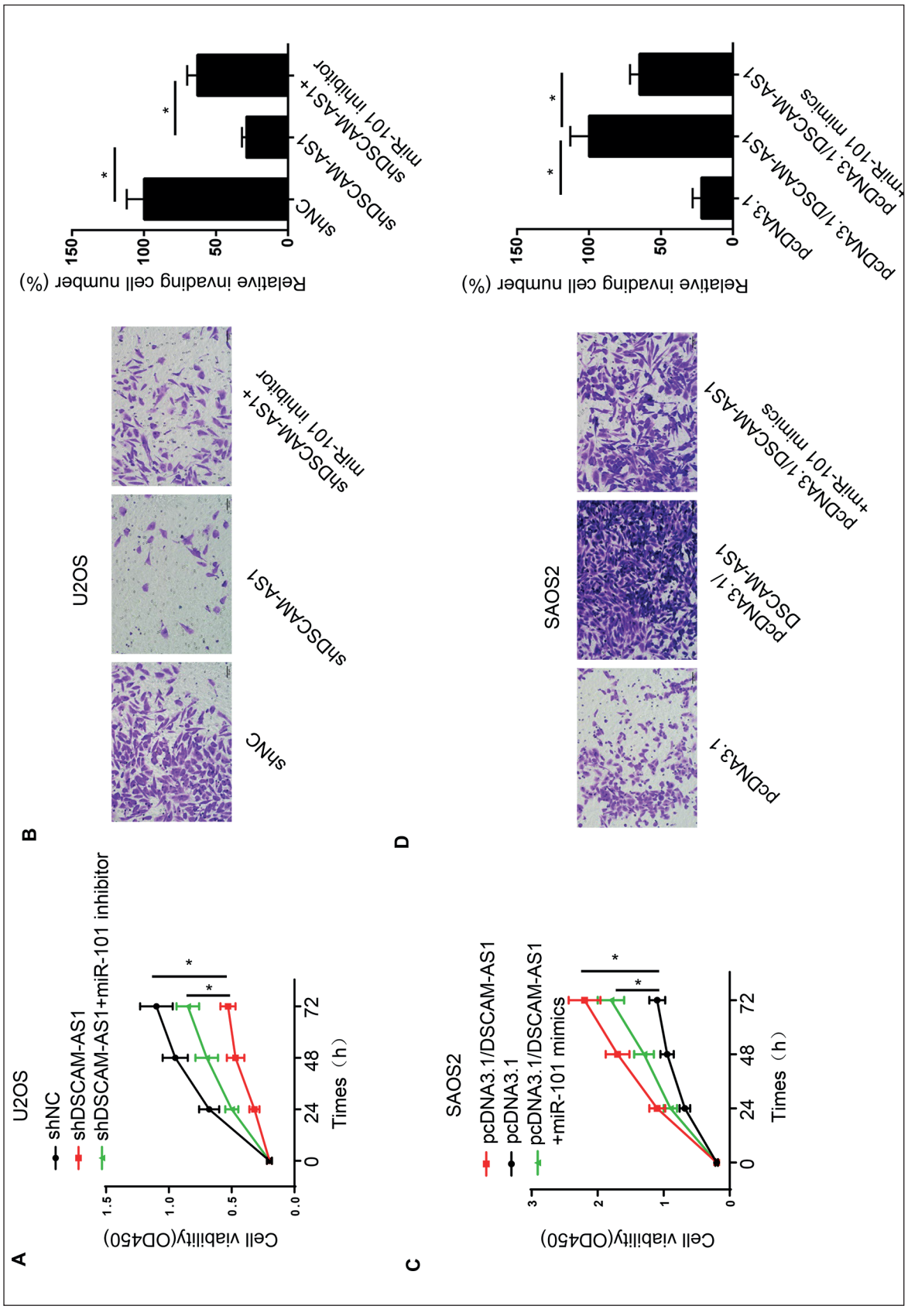

Figure 4. DSCAM-AS1 promoted cell proliferation and invasion by sponging miR-101 in OS cells. **A,** MTT assay showed the cell viability of U2OS transfected with shNC, shDSCAM-AS1, and shDSCAM-AS1 + miR-101 inhibitor. **B,** Tranwell assay showed the invasion ability of U2OS transfected with shNC, shDSCAM-AS1, and shDSCAM-AS1 + miR-101 inhibitor (magnification x40). **C,** MTT assay showed the cell viability of SAOS2 transfected with pcDNA3.1, pcDNA3.1/DSCAM-AS1 + miR-101 mimics. **D,** Tranwell assay showed the invasion ability of SAOS2 transfected with pcDNA3.1, pcDNA3.1/DSCAM-AS1 + miR-101 mimics (magnification ×40). *$p<0.05$.

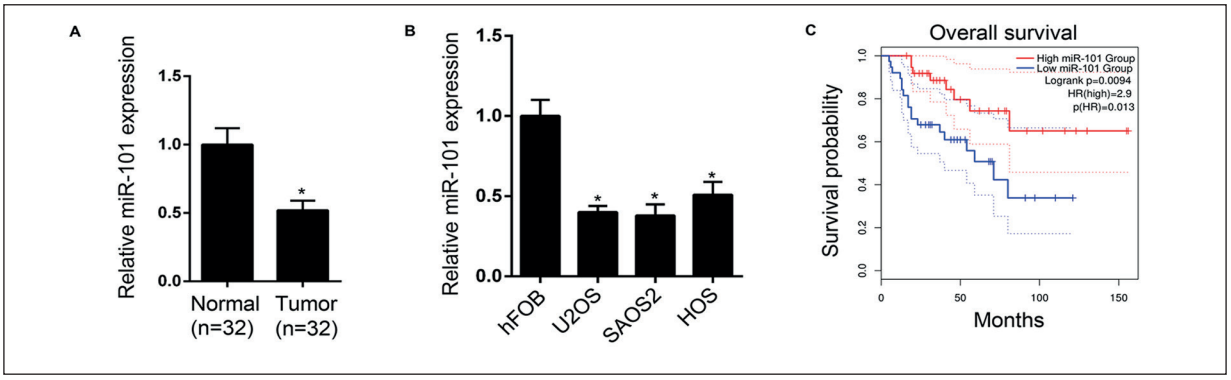

Figure 5. High expression of miR-101 in OS patients predicted favorable prognosis. **A,** RT-qPCR showed the relative miR-101 expression in OS tissues (n=32) and normal tissues (n=32). **B,** The expression of miR-101 in OS cell lines (U2OS, SAOS2, MG63, HOS) and human osteoblast cells (hFOB) was measured by RT-qPCR. **C,** Kaplan-Meier survival analysis showed the correlation between miR-101expression and prognosis of OS patients. *p<0.05.

MTT and transwell assays showed that the attenuated cell proliferation and invasion caused by DSCAM-AS1 knockdown could be reversed by the silencing of miR-101 in U2OS cells (Figure 4A and B). By contrast, the overexpression of DSCAM-AS1 promoted the proliferation and invasion of SAOS2 cells, while the promotion was reversed by overexpression of miR-101 (Figure 4C and D). In sum, the above data demonstrated that DSCAM-AS1 regulated OS progression by sponging miR-101.

High Expression of MiR-101 Predicted Favorable Prognosis in OS

RT-qPCR assay was employed to detect miR-101 expression in OS tissues and cell lines. The results demonstrated that miR-101 was highly expressed in OS tissues and cell lines compared with that in normal tissues and human osteoblast cells (Figure 5A and B). Kaplan-Meier analysis indicated that patients with high miR-101 expression had a longer survival time than those with low miR-101 expression (Figure 5C). In summary, the high expression of miR-101 predicted a favorable prognosis.

Discussion

LncRNA plays a vital role in the pathological activities of cancerous cells, such as proliferation, apoptosis, migration, and invasion. Notably, lncRNA PVT1 promoted proliferation and inhibits apoptosis in non-small cell lung cancer cells by regulating the miR-145-5p/ITGB8 axis[18]. LncRNA TP73-AS1 acted as an oncogenic gene to accelerate cell proliferation, migration, and invasion in OS[19]. In the current research, we demonstrated that DSCAM-AS1 expression was upregulated in OS tissues and cells, and high expression of DSCAM-AS1 indicated a poor prognosis. In addition, the inhibition of DSCAM-AS1 attenuated OS progression by inhibiting cell viability and invasion, and overexpression of DSCAM-AS1 dramatically promoted OS progression. Thus, our findings suggested that DSCAM-AS1 promoted the viability and invasion of OS cells.

LncRNAs interact with miRNAs by acting as competitive endogenous RNA (ceRNA) to regulate the development and progression of tumors. LncRNA MIR4435-2HG promoted melanoma progression by serving as a ceRNA for miR-802[20]. LncRNA RC3H2 promoted tumorigenesis of oral squamous cell carcinoma by acting as a ceRNA against miR-101-3p and upregulating EZH2[21]. LncRNA PSMB8-AS1 functioned as a ceRNA in regulating DDIT4 by competitively binding to miR-22-3p in glioblastoma[22]. In addition, miR-101 has been reported[23-15] to be associated with cancer regulation, such as breast cancer, hepatocellular carcinoma and bladder cancer. Consistent with these studies, miR-101 was confirmed to be a target gene of DSCAM-AS1, and miR-101 was negatively correlated with DSCAM-AS1 in OS tissues. In addition, we found that DSCAM-AS1 regulated OS progression by sponging miR-101.

Conclusions

The results of the present research reported the potential molecular mechanisms of DSCAM-AS1 in the tumorigenesis of OS. The data of the present study demonstrated for the first time that DSCAM-AS1 contributed to OS progression by regulating miR-101. The findings provide a better understanding for treating OS by using a DSCAM-AS1-targeted approach. Our study demonstrated that DSCAM-AS1 accelerated OS cell proliferation and invasion by modulating miR-101, which might provide a new therapy direction for OS treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Availability of Data and Materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

The investigation was approved by the Ethics Committee of Changzhou Second People's Hospital Affiliated to Nanjing Medical University.

Authors' Contribution

CY and YM designed the study. NX and WJ performed the experiments. CY and HZ analyzed the data and prepared the figures. CY and YM drafted the manuscript. All authors approved this manuscript.

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